Interleukin-10 Promoter Polymorphisms and Susceptibility to Skin Squamous Cell Carcinoma After Renal Transplantation

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After organ transplantation, susceptibility to cancer is multifactorial, especially for skin carcinomas. Risk factors may include genetic susceptibilities, such as the control of cytokine production. Interleukin-10 is a cytokine that is implicated in tumorigenesis, and it has been shown that polymorphisms in its gene promoter correlate with differential amounts of production. The aim of this study was to investigate a possible association between interleukin-10 gene promoter polymorphisms and the occurrence of skin carcinomas after renal transplantation. Seventy kidney transplant recipients who developed a squamous cell carcinoma or a basal cell carcinoma were examined for polymorphisms in the interleukin-10 gene promoter using polymerase chain reaction based methods. Single base pair mutations were studied at positions –1082, –819, and –592. These patients were compared to 70 healthy controls and to 70 matched renal transplant recipients without cancer. The interleukin-10 secretion capability was tested in a subgroup of 40 of these patients by *in vitro* stimulation of peripheral mononuclear cells. Interleukin-10 genotypes and haplotypes were differently distributed in kidney transplant recipients who developed a skin carcinoma, but especially a squamous cell carcinoma, with an increased frequency of the GCC haplotype and a decreased frequency of the ATA haplotype. Subsequently, we found a shift in the predicted phenotypes from the low production phenotype to the high production phenotype. Secretion of interleukin-10 was strongly correlated to the production predicted phenotype, and tended to be higher in patients who developed a squamous cell carcinoma than in the others. These results indicate that interleukin-10 gene polymorphisms and interleukin-10 production capability may contribute to the development of skin squamous cell carcinomas after renal transplantation. ***Key words: gene polymorphism/interleukin-10/renal transplantation/skin carcinomas. J Invest Dermatol 120:99–103, 2003***

Malignancies occur in at least 20% of transplant recipients within 20 y after grafting. This risk reaches 40% in certain studies (London *et al*, 1995). Among them, skin carcinomas account for up to 50%. Some viruses, such as papillomaviruses, Epstein Barr virus, and human herpesvirus-8, are involved in the pathogenesis of post-transplant tumors, especially in the cases of skin tumors, B-cell lymphomas, and Kaposi's sarcomas. The conjunction of impaired immune defenses and viral infections seems to be the trigger of tumorigenesis after organ transplantation. Heavy immunosuppression, use of polyclonal and monoclonal antibodies, high doses of cyclosporinee, and splenectomy have been linked to a higher rate of cancer. Other clinical risk factors for the occurrence of these malignancies have also been identified. These are mainly age at transplantation, duration of transplantation, sun exposure, and smoking. Genetic risk factors are suspected although less documented. The male gender has been suspected to be a risk factor and Kaposi's sarcoma affects mainly people from the Mediterranean area. In regard to skin carcinomas, human leukocyte antigen polymorphism (Bouwes Bavinck *et al*, 1991) and genetic variations in the glutathione S-transferase (Marshall *et al*, 2000) could confer susceptibility.

Levels of cytokine production seem critical during autoimmune disorders (Bouma *et al*, 1996), tumorigenesis (Zheng *et al*, 1996), and organ transplantation (Awad *et al*, 1998). Genetic variations that affect the production of cytokines could have an important effect on tumorigenesis after organ transplantation. Interleukin-10 (IL-10) is produced primarily by T helper type 2 lymphocytes and downregulates the Th_1 pathway. IL-10 inhibits macrophage-dependent antigen presentation; IL-10 induces T cell energy and downregulates major histocompatibility complex class II expression. IL-10 might therefore play a role in the induction of antigen-specific tolerance (Mosmann, 1994; Groux *et al*, 1996; Kundu and Fulton, 1997). Moreover, ultraviolet-induced DNA damage, which triggers the transformation of keratinocytes, increases the production of IL-10 (Nishigori *et al*, 1996).

Polymorphisms in cytokine gene promoters alter the production of mRNAs, and hence the amount of proteins. The gene encoding for IL-10 has been mapped on chromosome 1. Several polymorphisms that influence IL-10 production have been described in the IL-10 gene promoter region (Turner *et al*, 1997; Eskdale *et al*, 1998; D'Alfonso *et al*, 2000). In fact, three bi-allelic polymorphisms were found to be in strong linkage desequilibrium. Three single base pair mutations have been described: a G to A substitution at position –1082, a C to T substitution at position –819, and a C to A substitution at position –592. Three out

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of the eight possible haplotypes, i.e., GCC, ACC, and ATA, segregate in the general population (Turner *et al*, 1997).

In this study, we investigated a possible relationship between IL-10 gene polymorphisms and the occurrence of skin carcinomas following renal transplantation.

MATERIALS AND METHODS

Patients Among a cohort of 879 consecutive renal transplant recipients grafted in our center from 1979 to 1999, 87 developed at least one skin carcinoma. DNA was available for 70 of them who were all included in the genetic study. All the patients gave their formal agreement for a genomic study but no specific ethical approval was required, as the Ministry of Health has given our center authorization for genetic studies. These 70 patients were compared to 70 normal controls (healthy volunteers matched for gender) and to 70 transplant recipients without any cancer (unaffected patients). The latter were matched for year of transplantation, type of immunosuppression, gender, and age at grafting. Every transplant recipient is given repeated information on the danger of ultraviolet exposure and is asked to attend a yearly skin examination by a dermatologist. The tumors were 40 squamous cell carcinomas (SCC) and 30 basal cell carcinomas (BCC). The diagnosis was confirmed by histopathology in all cases. Patients with an SCC or a BCC were compared to controls, to unaffected patients, and between them. We analyzed the distributions of the genotypes and of the haplotypes. We also classified the predicted level of cytokine production according to their polymorphisms. GCC homozygous patients were considered as high producers (i.e., GCC/GCC genotype), GCC heterozygous patients as intermediate producers (i.e., GCC/ATA and GCC/ACC genotypes), and GCC negative patients as low producers (i.e., ATA/ATA, ACC/ATA, and ACC/ACC genotypes) (Turner *et al*, 1997).

Preparation of genomic DNA Genomic DNA from whole fresh peripheral mononuclear cells was obtained by a phenol chloroform extraction and ethanol precipitation. Unless specified, polymerase chain reaction (PCR) amplification of 250 ng DNA was done with 1.25 University of Thermus Aquaticus DNA polymerase (Gibco BRL, Carlsbad, CA) in 20 mM Tris–HCl containing 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTP, and 0.5 μM of each primer.

Genotyping IL-10 gene promoter polymorphisms We performed a first amplification with 0.025 μM primers surrounding the three positions, –1082, –819, and –592. The sense primer was 5′-ATC CAA GAC AAC ACT ACT AA-3′ and the antisense primer was 5′-TAA ATA TCC TCA AAG TTC C-3′. We proceeded to a second amplification for each position. The –1082 position was analyzed by a nested PCR restriction fragment length polymorphism. Fragments of the previous PCR were amplified with a 5′-ATC CAA GAC AAC ACT ACT AA-5′ sense primer and a 5′-GTG GAA GAA GTT GAA ATA AC-3′ antisense primer. Fragments were then digested with *Mnl-1* for a restriction fragment length polymorphism analysis and run on an 8% polyacrylamide gel. Allele A was characterized by two bands of 38 and 80 bp, and allele G by three bands of 24, 38, and 56 bp (**Fig 1**). For the –819 position, products of the first PCR were digested during 3 h at 55°C with *Mae-III* and run on an 8% polyacrylamide gel. Allele C was characterized by three bands of 79, 217, and 292 bp, and allele T by two bands of 79 and 509 bp (**Fig 2**). For the –592 position, products of the first PCR were digested during 3 h at 37°C with *Rsa-I* and run on an 8% polyacrylamide gel (**Fig 3**). Allele C was characterized by three bands of 42, 232, and 306 bp, and allele A by four bands of 42, 66, 232, and 240 bp.

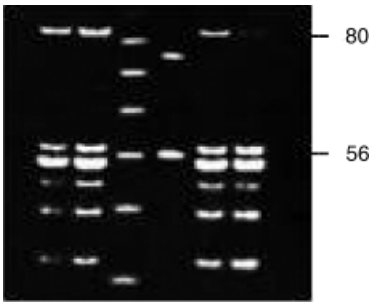

Figure 1. Polyacrylamide gel of the –1082 mutation in the IL-10 gene promoter. Allele A is indicated by the 80 bp band and allele G by the 56 bp band.

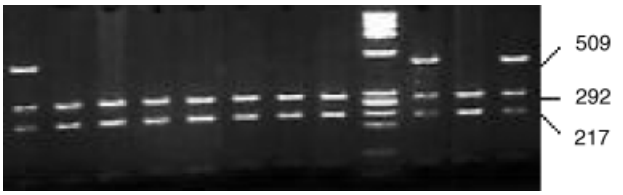

Figure 2. Polyacrylamide gel of the –819 mutation in the IL-10 gene promoter. Allele C is indicated by the 217 and 292 bp bands, allele T by the 509 bp band.

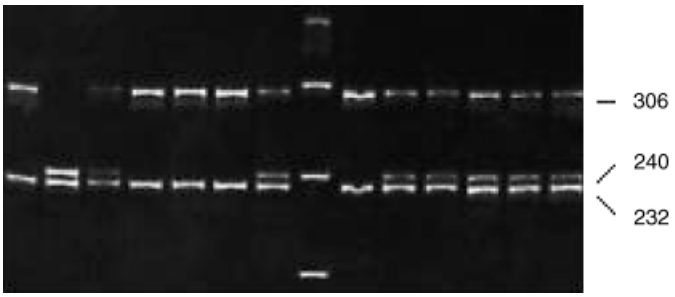

Figure 3. Polyacrylamide gel of the –592 mutation in the IL-10 gene promoter. Allele C is indicated by the 306 bp band and allele G by the 240 bp band.

IL-10 secretion Peripheral blood was drawn into heparinized tubes and mononuclear cells (PBMC) were harvested after Ficoll–Hypaque gradient centrifugation. PBMC were incubated at a concentration of 2×10^6 per ml in 24-well plates. Medium consisted of RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Stimulation was obtained by adding lipopolysaccharide (*Escherichia coli* extract, 055:B5, Sigma, St. Louis, MO) at a concentration of 1 μg per ml, during an 18 h incubation at 37°C, 5% CO_2. Supernatants were harvested for immediate freezing at −70°C. IL-10 was measured in supernatants by immunoassay with Quantikine HS (R&D Systems, Abingdon, UK).

Statistical analysis The data were analyzed using a χ^2 test; Fisher's exact test was applied when one of the expected values was less than 5; relative risks and odds ratios were calculated with their 95% confidence intervals. Continuous variables were analyzed by *t* test and ANOVA.

RESULTS

Patients' characteristics We tried to match the patients as well as possible in order to make patients with a skin carcinoma comparable to those without. In these two groups, we obtained the same distribution of gender (54 *vs* 49 males, $p = 0.33$), but age at grafting was higher in cancer patients (51 ± 11 *vs* 42 ± 11 y, $p = 0.0001$). Original renal diseases were similar in the two groups, as well as number of second transplants (10 in both groups), peak reactive antibodies level (15% *vs* 14%), induction with antithymocyte globulins (31% *vs* 34%), and total amount of immunosuppression. The latter was assessed by the number of treatments given to each patient, with antithymocyte globulins or boluses of methyl prednisolone, as either induction therapy or antirejection therapy. The mean transplant follow-up duration was 115 ± 61 mo in patients with a skin carcinoma and 120 ± 43 mo in unaffected patients (NS). SCC were discovered after a mean duration of 58 ± 39 mo, and BCC after 63 ± 35 mo (NS).

Distribution of the genotypes The IL-10 genotypes were unequally distributed between cancer patients, unaffected patients, and controls (**Table I**, $p = 0.001$). The distribution was

Table I. IL-10 genotypes

		Genotypes					
		ACC/ACC	ACC/ATA	ATA/ATA	GCC/ACC	GCC/ATA	GCC/GCC
Controls	70	4 (6)	12 (17)	8 (11)	18 (26)	15 (21)	13 (19)
Unaffected patients[a]	70	17 (24)	14 (20)	2 (3)	13 (19)	16 (23)	8 (11)
Cancer patients[b]	70	6 (9)	9 (13)	1 (1)	27 (39)	10 (14)	17 (24)
Squamous cell carcinomas[c]	40	2 (5)	3 (8)	0 (0)	17 (42)	8 (20)	10 (25)
Basal cell carcinomas	30	4 (13)	6 (20)	1 (3)	10 (33)	7 (23)	7 (23)

Point mutations at positions −1082 (A→G), −819 (T→C), and −592 (A→C) in the IL-10 gene promoter are responsible for the common GCC, ACC, and ATA combinations.

Results are given as numbers and percentages (in parentheses).

[a] $p = 0.016$ *vs* controls.

[b] $p = 0.006$ *vs* unaffected patients.

[c] $p = 0.003$ *vs* unaffected patients.

also different when cancer patients were compared to unaffected patients ($p = 0.006$), or when patients with an SCC were compared to unaffected patients ($p = 0.003$). Patients with a BCC did not differ from unaffected patients or from patients with an SCC. We note that the distribution of these genotypes was different between unaffected patients and controls but with a borderline significance ($p = 0.016$).

Distribution of the haplotypes These differences were easier to acknowledge with the distribution of the haplotypes (**Table II**). Cancer patients, unaffected patients, and controls were altogether different ($p = 0.0009$). This was due to the lower frequency of the ATA haplotype in kidney transplant recipients with a skin carcinoma (15%), either SCC (14%) or BCC (17%), than in unaffected patients (24%) and in controls (31%). In parallel, we found a higher frequency of the GCC haplotype in patients with a skin carcinoma (51%), either SCC (56%) or BCC (43%), than in unaffected patients (32%) and in controls (42%). The haplotype distribution was significantly different when cancer patients were compared to controls ($p = 0.007$) and to unaffected patients ($p = 0.006$), or when patients with an SCC were compared to unaffected patients ($p = 0.003$). Patients with a BCC did not differ from unaffected patients, or from patients with an SCC.

Distribution of the production predicted phenotypes The production predicted phenotypes were also unequally distributed between cancer patients, unaffected patients, and controls ($p = 0.038$, **Table III**). The phenotype associated with a low production of IL-10 (GCC negative patients) was less frequent in cancer patients (23% *vs* 47% in unaffected patients), but only in the patients with an SCC (12%) and not in the case of patients with a BCC (37%). The phenotype associated with a high production of IL-10 (GCC homozygous patients) was more frequent in cancer patients (24% *vs* 11% in unaffected patients), either SCC (25%) or BCC (23%). The comparisons were significant between cancer patients and unaffected patients ($p = 0.006$) and between patients with an SCC and unaffected patients ($p = 0.0009$). Once again, the patients with a BCC did not differ from unaffected patients.

In cancer patients with an SCC, the frequency of the low predicted phenotype was compared to that of high and intermediate predicted phenotypes. The odds ratio was 0.16 (95% confidence interval, 0.06–0.42) and the relative risk was 0.27, indicating a protective effect of the low predicted phenotype. When the frequency of the high predicted phenotype was compared to that of intermediate or low predicted phenotypes, we found an odds ratio of 2.58 (95% confidence interval, 1.05–7.04). The relative risk was 1.7, indicating a deleterious effect of the high predicted phenotype.

***In vitro* secretion of IL-10** The IL-10 production capacity was investigated in a subpopulation of 40 of these transplant recipients. Secretion of IL-10 was compared according to the production predicted phenotypes as defined by genetic analysis. After stimulation by lipopolysaccharide, the secretion of IL-10 was significantly different in the high, medium and low predicted phenotypes with 1311 ± 585 pg per ml, $736 + 536$ pg per ml, and 449 ± 250 pg per ml, respectively (**Fig 4**) (ANOVA, $p = 0.0002$). These data indicated a strong relationship between gene polymorphisms and IL-10 secretion in individuals. Of the 40 patients 16 were unaffected patients, 17 were patients with an SCC, and seven were patients with a BCC. The mean production of IL-10 was 649 ± 491 pg per ml in unaffected patients, 917 ± 685 pg per ml in patients with an SCC, and 699 ± 282 pg per ml in patients with a BCC (**Fig 5**). Although IL-10 secretion was higher in the patients with an SCC, the difference did not reach statistical significance.

Table II. IL-10 haplotypes

		Haplotypes		
		GCC	ACC	ATA
Controls	140	59 (42)	38 (27)	43 (31)
Unaffected patients[a]	140	45 (32)	61 (44)	34 (24)
Cancer patients[b]	140	71 (51)	48 (34)	21 (15)
Squamous cell carcinomas[c]	80	45 (56)	24 (30)	11 (14)
Basal cell carcinomas	60	26 (43)	24 (40)	10 (17)

The three haplotypes that segregate in the general population (GCC, ACC, and ATA) are indicated.

Results are given as numbers and percentages (in parentheses).

[a] $p = 0.015$ *vs* controls.

[b] $p = 0.006$ *vs* unaffected patients and 0.007 *vs* controls.

[c] $p = 0.003$ *vs* unaffected patients.

Table III. Predicted phenotypes of IL-10 production

		Predicted phenotypes		
		High	Intermediate	Low
Controls	70	13 (19)	33 (47)	24 (34)
Unaffected patients	70	8 (11)	29 (41)	33 (47)
Cancer patients[a]	70	17 (24)	37 (53)	16 (23)
Squamous cell carcinomas[b]	40	10 (25)	25 (63)	5 (12)
Basal cell carcinomas	30	7 (23)	12 (40)	11 (37)

GCC homozygous patients were considered as high producers, GCC heterozygous patients as intermediate producers, and GCC negative patients as low producers.

Results are given as numbers and percentages (in parentheses).

[a] $p = 0.006$ *vs* unaffected patients.

[b] $p = 0.0009$ *vs* unaffected patients.

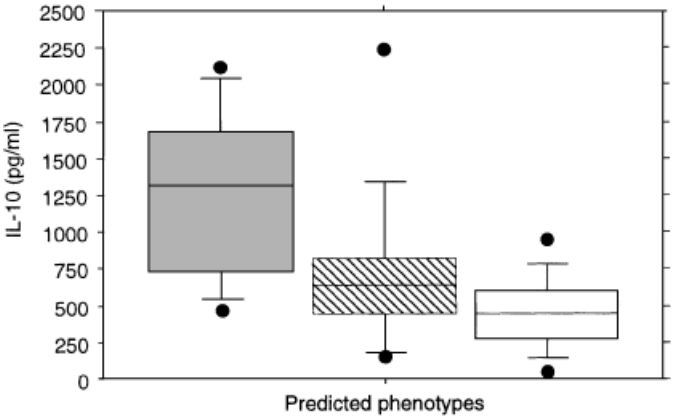

Figure 4. IL-10 secretion and production predicted phenotypes. Box-plot representation of IL-10 concentration in the culture supernatant of PBMC after lipopolysaccharide stimulation is given for high (*gray*), medium (*dashed*), and low (*white*) predicted phenotypes.

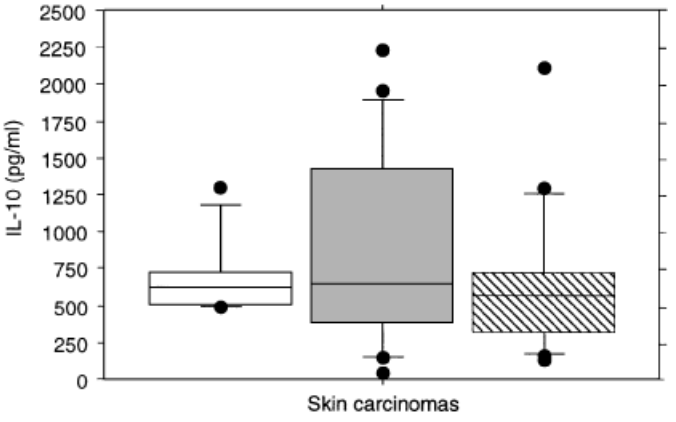

Figure 5. IL-10 secretion and skin carcinomas. Box-plot representation of IL-10 concentration in the culture supernatant of PBMC after lipopolysaccharide stimulation is given for BCC patients (*white*), SCC patients (*gray*), and unaffected patients (*dashed*).

DISCUSSION

Cancer is a potentially remediable aberration in the continuum of normal cell regulation. A great number of cancers are associated with human viruses, although the virus infection is not sufficient *per se*. Immunosuppression, inhibition of apoptosis, and tolerance to tumor antigens may contribute to tumorigenesis after transplantation (Birkeland and Bendtzen, 1996). Because of their immunomodulatory effects and their action on viral open reading frames, cytokines are supposed to play a role in tumorigenesis. As there is growing evidence for a genetic basis of cancer (Haber and Fearon, 1998), much attention is being paid to the genetic control of cytokine production.

We should be aware of the limits of investigations on association with genetic polymorphisms. The phenotypic heterogeneity of the disease, the insufficient number of cases, the possible neutral effect of the studied allele, and the missing of a truly relevant polymorphism are the pitfalls of these studies (Gambaro *et al*, 2000). Many other polymorphisms have been described in association with skin cancer. Glutathione S-transferase is a group of antioxidant enzymes that protect against ultraviolet-mediated DNA damage. A polymorphism at the glutathione S-transferase locus GSTM3 was found in association with cutaneous BCC in immunocompetent people (Yengi *et al*, 1996), and also in immunocompromised patients (Marshall *et al*, 2000). The E6 oncoprotein derived from human papillomaviruses binds to and induces the degradation of the tumor suppressor protein p53. A common polymorphism that occurs in the p53 sequence, which results in the presence of an arginine instead of a proline at position 72, seems to favor human papillomavirus-associated cancers (Storey *et al*, 1998), although other studies have challenged this hypothesis (Marshall *et al*, 2000; O'Connor *et al*, 2001). A polymorphism in tumor necrosis factor α microsatellite has been related to an increased risk of multiple BCC (Hajeer *et al*, 2000). Lastly, the melanocortin-1-receptor gene variants, which are known to be associated with melanoma, have been found to be associated with nonmelanoma skin cancer (Bastaiens *et al*, 2001; Box *et al*, 2001).

In this study, the patients' characteristics were the same except for a 10 y difference in age at grafting. Although we tried to match the patients as well as possible, choosing several parameters made us fail this goal. Gender distribution was similar as well as the type and the amount of immunosuppression, which is a major factor for the occurrence of cancer after organ transplantation. We did not assess sun exposure, but all the patients lived in a common geographic area. Counseling and dermatologic examination were the same for all transplant recipients. The distributions of the genotypes and haplotypes between unaffected patients and controls were slightly unequal but this does not jeopardize our results. Indeed, the genotypes and the three main haplotypes, i.e., GCC, ACA, and ATA, were not equally distributed when comparison was done between unaffected patients and cancer patients, with a shift from the ATA to the GCC haplotype. Hence, we found a relationship between IL-10 polymorphism and post-transplant cutaneous carcinomas. Of greater functional relevance, the low production phenotype was less frequent in cancer patients, whereas the high production phenotype was more frequent. All these results were in fact obtained only in the group of patients with an SCC. Patients with a BCC were fewer but these results may indicate a difference in the response to IL-10 between SCC and BCC. As a whole, these data indicate that IL-10 production might favor the occurrence of carcinoma in transplanted patients, and especially of SCC. We did not examine other polymorphisms that exist in the IL-10 gene and that also affect IL-10 production (Eskdale *et al*, 1996; D'Alfonso *et al*, 2000). They could be implicated in post-transplant skin carcinomas.

The actual secretion of IL-10 was tested by a functional assay in a subpopulation of 40 transplant recipients. These patients were included only on the basis that it was possible to harvest fresh blood from them. Significant differences were found between high, medium, and low predicted producers for IL-10, providing further evidence that variation in the protein production within individuals may result from genetic changes in cytokine genes. We also compared the IL-10 secretion among cancer and unaffected patients. We did not find a statistical difference in these maybe few patients but individuals with an SCC produced much more IL-10 than the others. The distribution of IL-10 secretion appeared to be wide in patients with an SCC (**Fig 5**) as well as in individuals of the high production phenotype (**Fig 4**), which could explain this lack of significance.

Due to its immunosuppressive and anti-inflammatory properties, it has been hypothesized that IL-10 may contribute to the escape of tumor cells from immune surveillance and favor tumor growth. Many studies support a role for IL-10 in malignant cutaneous lesions. IL-10 levels are higher in the serum of patients with advanced melanoma than in controls (Nemunaitis *et al*, 2001) and metastatic melanoma cells preferentially produce IL-10 (Dummer *et al*, 1996). Genotypes associated with high levels of IL-10 expression have been showed to be protective against cutaneous malignant melanoma (Howell *et al*, 2001). The expression of IL-10 mRNA is downregulated in warts (Jackon *et al*, 1996) and the production of IL-10 and its subsequent shift to a type 2 cytokine production is associated with more extensive papillomavirus infection in women with cervical carcinomas (Clerici *et al*, 1997). Nevertheless, experiments in animals and humans yielded contradictory results regarding the effects of IL-10. For example, the transfection of IL-10 in melanoma cells (Huang *et al*, 1999) or mammary tumor cells (Kundu *et al*, 1996) resulted in a loss of

metastasis and inhibition of tumor growth. Closer to skin carcinomas, IL-10 increased Th_1 cytokine production and papillomavirus-specific cytotoxic T lymphocytes in cervical cancer patients (Santin *et al*, 2000). Therefore, the perception of IL-10 as solely an immunosuppressive cytokine is being challenged. Investigations have also been carried out during benign cutaneous disorders such as psoriasis. They too led to contradictory results. No association was found with point mutations at position −1082 (Reich *et al*, 1999), whereas others found that polymorphism in the microsatellite regions contributes to susceptibility to the disease (Asadullah *et al*, 2001).

In this study in kidney transplant recipients with skin carcinomas, we found that IL-10 genotypes and haplotypes were differently distributed, with an increased frequency of the GCC haplotype and a decreased frequency of the ATA haplotype. The distribution of the predicted phenotypes was shifted from the low IL-10 production phenotype to the high IL-10 production phenotype. These results affected patients with an SCC, but not those with a BCC. *In vitro* secretion of IL-10 by mononuclear cells was strongly correlated to the production predicted phenotype, strengthening the known influence of genetic polymorphism on cytokine production capability. We also found a trend for a higher secretion of IL-10 in the patients with an SCC, although great caution is required in interpreting this last result. We conclude that IL-10 production capability might make individuals more susceptible to SCC after organ transplantation.

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